EXHIBIT 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Cell Populations which Co-Express CD49c and CD90	**Confirmation No.:** 4326
Application No.: 09/960,244	**Art Unit:** 1651
Filed: Sept. 21, 2001	**Examiner:** Leon B. Lankford, Jr.
Assignee: Neuronyx Inc.	**Atty. Docket:** 2560.0020000

Declaration of Gene Kopen Under 37 C.F.R. § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, Gene C. Kopen, declare and state as follows:

Add this to appropriate section:

A. INTRODUCTION AND SUMMARY

1. I received a B.A. degree in Social & Behavioral Sciences from the University of South Florida (Tampa, FL) and a Ph.D. in Molecular Pathobiology from Drexel College of Medicine (Philadelphia, PA). My training continued as a post-doctoral fellow at Hahnemann University (Philadelphia, PA). A copy of my curriculum vitae is attached as **Exhibit A**.

2. I am a co-inventor of the presently claimed invention (U.S. Patent Application No. 09/960,244). I am also currently employed at Neuronyx Incorporated (assignee of the presently claimed invention), where I hold the position of Senior Scientist. My work

involves, in part, training research and development staff, developing and implementing methods for large scale expansion of human stem cells, providing support and oversight in product development and testing, initiating and cultivating external research collaborations, and managing in-house pre-clinical studies supporting clinical development.

3. I am familiar with the above-identified patent application and pending claims as well as the March 7, 2007 Office Action (Paper No. 20070116) issued in relation to this application.

4. I have been told by attorneys for Neuronyx that the specification of a patent application describes the claimed invention while the claims establish the scope of the invention. I understand that presently pending claims 14, 19-21 and 25-26 are directed to isolated cell populations derived from human bone marrow wherein greater than about 91% of the cells of the cell population co-express CD49c and CD90 and wherein the cell population has a doubling rate of less than about 30 hours.

5. I have been asked to give my opinion on whether the isolated cell populations described and claimed in the present patent application are the same, or are essentially the same, as cell populations described in a patent issued to Furcht *et al.*, U.S. Patent No. 7,015,037 (hereinafter the "Furcht patent").

6. It is my opinion that the cells described and claimed in the present patent application are not the same, nor are they essentially the same, as the cell populations as described in the Furcht patent. In addition to the description provided in the present patent application, this opinion is also based on additional experimental evidence described herein as well as documented knowledge commonly known to those of ordinary skill in the art.

7. I have also been asked to give my opinion on statements made in a declaration by Robert J. Deans, Ph.D. (dated Mar. 10, 2005; submitted in U.S. Patent Application No. 10/048,757) with respect to the experiments performed as described therein. In particular, I

have been asked to give my opinion on how experiments performed as described in the Declaration by Robert Deans relate, or do not relate, to the cells described in the present patent application.

8. It is my opinion that the conclusions asserted in the Declaration by Robert J. Deans (hereinafter "Deans") were based on an experimentally flawed approach to the questions asked and, therefore, the ultimate conclusions reached are incorrect. In particular, Deans concluded that the cells "prepared by the method of the Furcht '757 application" and the cells of the present invention are "essentially the same." *See*, Deans, page 8, item 11; *see also*, item 13. It is my opinion, based on the reasons and data described in the present declaration, that this conclusion by Deans is incorrect.

B. THE BONE MARROW-DERIVED CELL POPULATIONS IN THE FURCHT PATENT HAVE A DIFFERENT PHENOTYPE COMPARED TO THE CELL POPULATIONS DESCRIBED IN THE PRESENT PATENT APPLICATION

Attached **Exhibit B** shows fluoresence activated cell-sorting (FACs) analysis of human bone marrow-derived cell populations isolated and cultured as described in the present patent application. All of the FACs data shown in **Exhibit B** was generated at Neuronyx Inc. (1 Great Valley Parkway, Suite 20, Malvern, PA), between March 2004 and March 2007. Each of these FACs experiments were carried out by someone under my direct supervision and control.

The vertical axis in **Exhibit B** shows the number of population doublings (PD) that cells from a single human donor have undergone (from 18.8 to 51.7 population doublings). Along the horizontal axis, **Exhibit B** shows measurement of cell surface expression of four different cell-surface markers (CD10, CD44, HLA Class-1, and β 2-Microglobulin) over the course of 51.7 population doublings. Notably, expression of these four markers remains constant from cell population doubling 18.8 (the earliest measurement) through cell population doubling 51.7 (the latest measurement). The cells used to generate the data in

Exhibit B were originally derived from a single human donor in February of 2004 using the same methods described in U.S. Patent Application No. 09/960,244 (filed on Sept. 21, 2001).[FN[1]] At the bottom of **Exhibit B** are brief summaries of relevant sections of the Furcht patent. These summarized sections are also addressed in further detail in the present declaration.

1. CD10 EXPRESSION

The isolated bone marrow-derived cell populations of the present invention are consistently negative for expression of the cell-surface protein CD10. In contrast, the bone marrow-derived cell populations taught in the Furcht patent (referred to as MASCs or "multipotent adult stem cells") are consistently described as positive for expression of CD10.

a) The FACs analysis in **Exhibit B** shows that isolated cell populations of the present invention are essentially negative for cell-surface expression of CD10 from at least 18.8 to at least 51.7 cell doublings. *See*, **Exhibit B**, col. 1, labeled "**CD10**", rows 1-5 corresponding to 18.8, 27.2, 35.2, 43.9, 51.7 population doublings (PD). A Raji cell line CD10 positive control was run in parallel with the CD10 FACs analysis (data shown in attached **Exhibit C**). This positive control demonstrates that CD10 was not detected in the isolated cells of the present invention due to lack of cell-surface expression, and not due to a failure in the experimental procedures.

b) In contrast, in the SUMMARY OF THE INVENTION the Furcht patent asserts that "The present invention provides an isolated multipotent mammalian stem cell"

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[1] In particular, the cells were initially isolated according to the method described in Example 1 of the present application, wherein: a) a bone marrow aspirate was obtained from a healthy adult human volunteer; b) the aspirate was mixed with an ammonium chloride buffer to lyse the red blood cells; c) the mononuclear cells were pelleted and resuspended in complete media; d) the cells were seeded into tissue culture-treated containers at ~50,000 cells/cm^2; and, e) the cells were incubated at 37°C in an atmosphere consisting of 5% carbon dioxide, 5% oxygen, and 90% nitrogen/air until adherent colony forming units were obtained. Thereafter, the cells were expanded and passaged according to the methods described in Examples 3-4 of the present application, wherein the cells were cultured under the same growth conditions described above while being re-seeded at a density of ~30 cells/cm^2 with each cell passage.

that is, *in particular*, surface antigen positive for CD10. *See*, Furcht patent, col. 5, lines 58-67. In col. 16, lines 40-48, the Furcht patent describes FACS (fluorescence-activated cell sorting) analysis of isolated human MASCs demonstrating that these cells express CD10 after 22-25 cell doublings. In col. 45, lines 43-51, the Furcht patent describes FACS analysis of isolated human MASCs demonstrating that these cells "stained highly positive with antibodies against CD10" after 10-12 cell doublings and that "[t]he MASC phenotype remained unchanged for >30 cell doublings". The Furcht patent also describes isolated human MASCs after 22 and 26 cell doublings as positive for CD10 mRNA expression. *See*, *Id.* at col. 16, line 62 to col. 17, line 7; and, col. 53, lines 42-56.

2. *CD44 EXPRESSION*

The isolated bone marrow-derived cell populations of the present invention are consistently positive for expression of the cell-surface protein CD44. In contrast, the bone marrow-derived cell populations taught in the Furcht patent are generally described as negative for expression of CD44.

a) The FACs analysis in **Exhibit B** shows that isolated cell populations of the present invention are positive for cell-surface expression of CD44 from at least 18.8 to at least 51.7 cell doublings. *See*, **Exhibit B**, col. 2, labeled "**CD44**", rows 1-5 corresponding to 18.8, 27.2, 35.2, 43.9, 51.7 population doublings (PD).

b) In contrast, in the SUMMARY OF THE INVENTION the Furcht patent teaches that "The present invention provides an isolated multipotent mammalian stem cell that is surface antigen negative for CD44... In particular, the cell may be surface antigen negative for...CD44..." *See*, Furcht patent, col. 5, lines 58-66.

In the DETAILED DESCRIPTION of the Invention the Furcht patent teaches:

> The phenotype of stem cells derived from different organs with "plasticity" is similar (CD45$^-$ CD44$^-$ HLA-DR$^-$ HLA-calss I $^-$ [sic] oct3/4 mRNA$^+$ and hTRT$^+$).

Id. at col. 14, lines 7-15 (emphasis added). Within this same section of the specification the Furcht patent teaches:

> The present inventors have shown that MASCs cultured at low density express the LIF-R, and these cells do not or minimally express CD44 whereas cells cultured at high density, that have characteristics of MSC [sic], loose [sic] expression of LIF-R but express CD44. [**FN**[2]]

Id. at col. 15, lines 43-47 (emphasis added).

In a section of the specification describing analysis of "Human MASCs" the Furcht patent teaches:

> Immunophenotypic analysis by FACS of human MASCs obtained after 22-25 cell doublings showed that cells do not express CD31, CD34, CD36, CD38, CD45, CD50, CD62E and -P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek; and express low levels of CD44, HLA-class I, and β2-microglobulin, but express CD10, CD13, CD49b, CD49e, CDw90, Flk1 (N>10).
>
> Once cells undergo >40 doublings in cultures re-seeded at $2x10^3/cm^2$, the phenotype becomes more homogenous and no cell expressed HLA-class-I or CD44 (n=6). When cells were grown at higher confluence, they expressed high levels of Muc18, CD44, HLA-class I and β2-microglobulin, which is similar to the phenotype described for MSC(N=8) (Pittenger, Science (1999) 284: 143 147). [**FN**[3]]

Id. at col. 16, lines 42-55 (emphasis added). With respect to cells isolated from murine bone marrow, the Furcht patent also teaches:

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[2] In contrast to the cells described in the Furcht patent, the isolated cells of the present invention consistently express CD44, even when cultured at low density. In evidence, the cells analyzed by FACs as shown in Exhibit B were cultured at low density (*i.e.*, seeded at 30 cells/cm^2) and FACs analysis was performed on cells harvested from sub-confluent populations.

[3] In contrast, the isolated bone marrow-derived cells of the present invention consistently express CD44 even after more than 40 population doublings. *See*, Exhibit B, col. 2, rows 4 & 5 (showing positive CD44 expression at 43.9 and 51.7 population doublings).

> As for human cells, C57/BL6 MASCs cultured with EGF, PDGF-BB and LIF are CD44 and HLA-class-I negative, stain positive with SSEA4, and express transcripts for oct-4, LIF-R, Rox-1 and sox-2.

Id. at col. 18, lines 27-30 (emphasis added).

In the Examples section, Example 1 describes the "Isolation of MASCs from Bone Marrow Mononuclear Cells". Moreover, this section of the Furcht patent teaches:

> Immunophenotypic analysis by FACS of cells obtained after 10-12 cell doubling showed that cells did not express CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62-P, Muc18, cKit, Tie/Tek, and CD44... As was seen for cells cultured with 2% FCS without IGF, cells cultured in serum-free medium were HLA-class-I and CD44 negative, and could differentiate into all mesodermal phenotypes, as described below.

Id. at col. 45, lines 39-62 (emphasis added).

> When cells were plated on collagen-type-I or laminin instead of fibronectin, they expressed CD44 and HLA-DR, and could not be expanded beyond 30 cell doublings. [**FN**[4]] When EGF or PDGF were omitted cells did not proliferate and died, while increased concentrations of these cytokines allowed initial growth of MASC but caused loss of proliferation beyond 20-30 cell doublings. [**FN**[5]] Addition of higher concentrations of dexamethasone also caused loss of proliferation beyond 30 cell doubling. When cells were cultured with >2% FCS in the culture medium they expressed CD44, HLA-DR and HLA-class-I. Likewise, culture at high density (>8X10^3 cells/cm^2) was associated with the acquisition of CD44, HLA-DR and HLA-class-I and Muc-18, which is similar to the phenotype described for MASC. Culture at high density or with higher concentrations of FCS was also associated with loss of expansion capacity, and cells did not proliferate beyond 25-30 cell doublings. [**FN**[6]]

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[4] In contrast, the isolated bone marrow-derived cells of the present invention express CD44 without plating on collagen or laminin and they can be expanded well beyond 30 cell doublings. *See*, Exhibit B, col. 2, rows 4 & 5 (showing CD44 expression at 43.9 and 51.7 population doublings).

[5] In contrast, the isolated cells of the present invention continue to proliferate in excess of 50 cell doublings in the absence of EGF and PDGF.

[6] In contrast, the isolated cells of the present invention continue to proliferate in excess of 50 cell doublings in the presence of 10% fetal bovine serum.

Id. at col. 45, line 63 to col. 46, line 12 (emphasis added).

3. HLA-CLASS 1 EXPRESSION

The isolated bone marrow-derived cell populations of the present invention are consistently positive for expression of the HLA-Class 1 cell-surface molecule. In contrast, the bone marrow-derived cell populations taught in the Furcht patent are generally described as negative for expression of HLA-Class 1.

a) The FACs analysis in **Exhibit B** shows that isolated cell populations of the present invention are positive for expression of the HLA-Class 1 cell-surface molecule from at least 18.8 to at least 51.7 cell doublings. *See*, **Exhibit B**, col. 3, labeled "**HLA 1**", rows 1-5 corresponding to 18.8, 27.2, 35.2, 43.9, 51.7 population doublings (PD).

b) In contrast, in the SUMMARY OF THE INVENTION the Furcht patent teaches:

> The present invention provides an isolated multipotent mammalian stem cell that is surface antigen negative for CD44, CD45, and HLA Class I and II. The cell may also be surface antigen negative for CD34, Muc18, Stro-1, HLA-class-I and may be positive for oct3/4 mRNA, and may be positive for hTRT mRNA. In particular, the cell may be surface antigen negative for CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek, CD44, HLA-class 1...

Id. at col. 5, lines 58-66 (emphasis added).

In the DETAILED DESCRIPTION of the Invention the Furcht patent teaches:

> The phenotype of stem cells derived from different organs with "plasticity" is similar ($CD45^-$ $CD44^-$ HLA-DR^- HLA-calss I^- [sic] oct3/4 $mRNA^+$ and $hTRT^+$).

Id. at col. 14, lines 7-15 (emphasis added).

In a section of the specification describing analysis of "Human MASCs" the Furcht patent teaches:

> Immunophenotypic analysis by FACS of human MASCs obtained after 22-25 cell doublings showed that cells do not express CD31, CD34, CD36, CD38, CD45, CD50, CD62E and -P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek; and express low levels of CD44, HLA-class I, and β2-microglobulin, but express CD10, CD13, CD49b, CD49e, CDw90, Flk1 (N>10).
>
> Once cells undergo >40 doublings in cultures re-seeded at $2x10^3/cm^2$, the phenotype becomes more homogenous and no cell expressed HLA-class-I or CD44 (n=6). [**FN**[7]] When cells were grown at higher confluence, they expressed high levels of Muc18, CD44, HLA-class I and β2-microglobulin, which is similar to the phenotype described for MSC(N=8) (Pittenger, Science (1999) 284: 143 147). [**FN**[8]]

Id. at col. 16, lines 42-55 (emphasis added).

With respect to cells isolated from murine bone marrow, the Furcht patent also teaches:

> As for human cells, C57/BL6 MASCs cultured with EGF, PDGF-BB and LIF are CD44 and HLA-class-I negative, stain positive with SSEA4, and express transcripts for oct-4, LIF-R, Rox-1 and sox-2.

Id. at col. 18, lines 27-30 (emphasis added).

In the Examples section, Example 1 describes the "Isolation of MASCs from Bone Marrow Mononuclear Cells". This section of the Furcht patent teaches:

> Immunophenotypic analysis by FACS of cells obtained after 10-12 cell doubling showed that cells did not express CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62-P, Muc18, cKit, Tie/Tek, and CD44. Cells

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[7] As shown in Exhibit B, the isolated bone marrow-derived cells of the present invention consistently express CD44 even after more than 40 population doublings. *See*, Exhibit B, col. 2, rows 4 & 5 (showing positive CD44 expression at 43.9 and 51.7 population doublings).

[8] In contrast, the isolated bone marrow-derived cells of the present invention continue to express the HLA-Class 1 molecule in excess of 40 cell doublings. *See*, Exhibit B, col. 3, rows 4 & 5 (showing "HLA-1" expression at 43.9 and 51.7 population doublings).

> expressed no HLA-DR or HLA-class-I and expressed low levels of P2-microglobulin... When cells were cultured in serum-free medium, also supplemented with 10 ng/mL IGF, cell doubling was slower (>60b [sic]), but >40 cell doublings could be obtained. As was seen for cells cultured with 2% FCS without IGF, cells cultured in serum-free medium were HLA-class-I and CD44 negative, and could differentiate into all mesodermal phenotypes, as described below.

Id. at col. 45, lines 39-62 (emphasis added).

> When cells were plated on collagen-type-I or laminin in stead of fibronectin, they expressed CD44 and HLA-DR, and could not be expanded beyond 30 cell doublings. [**FN**[9]] When EGF or PDGF were omitted cells did not proliferate and died, while increased concentrations of these cytokines allowed initial growth of MASC but caused loss of proliferation beyond 20-30 cell doublings. [**FN**[10]] Addition of higher concentrations of dexamethasone also caused loss of proliferation beyond 30 cell doubling. When cells were cultured with >2% FCS in the culture medium they expressed CD44, HLA-DR and HLA-class-I. Likewise, culture at high density (>$8X10^3$ cells/cm^2) was associated with the acquisition of CD44, HLA-DR and HLA-class-I and Muc-18, which is similar to the phenotype described for MASC. Culture at high density or with higher concentrations of FCS was also associated with loss of expansion capacity, and cells did not proliferate beyond 25-30 cell doublings. [**FN**[11]]

Id. at col. 45, line 63 to col. 46, line 12 (emphasis added).

Moreover, the Furcht patent emphasizes the desirability of eliminating endogenous HLA Class-1 expression from isolated donor cells. In this regard, the specification teaches:

> 15. Approaches for transplantation to prevent immune rejection:
> a. universal donor cells: MASC can be manipulated to serve as universal donor cells for cell and gene therapy to remedy genetic or other diseases and

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[9] In contrast, the isolated bone marrow-derived cells of the present invention express HLA-Class 1 without plating on collagen or laminin and they can be expanded well beyond 30 cell doublings. *See,* Exhibit B, col. 2, rows 4 & 5 (showing CD44 expression at 43.9 and 51.7 population doublings).

[10] In contrast, the isolated cells of the present invention continue to proliferate in excess of 50 cell doublings in the absence of EGF and PDGF.

[11] In contrast, the isolated cells of the present invention continue to proliferate in excess of 50 cell doublings in the presence of 10% fetal bovine serum.

> to replace enzymes. Although undifferentiated MASC express no HLA-type I, HLA-type II antigens or beta-2 microglobulin, some differentiated progeny express at least type I HLA-antigens. MACS can be modified to serve as universal donor cells by eliminating HLA-type I and HLA-type II antigens, and potentially introducing the HLA-antigens from the prospective recipient to avoid that the cells become easy targets for NK-mediated killing, or become susceptible to unlimited viral replication and/or malignant transformation.

Id. at col. 28, line 61 to col. 29, line 17 (emphasis added).

4. β2-MICROGLOBULIN EXPRESSION

The isolated bone marrow-derived cell populations of the present invention are consistently positive for expression of β 2-Microglobulin (β2-M). In contrast, the bone marrow-derived cell populations taught in the Furcht patent are generally described as negative for expression of β2-M.

a) The FACs analysis in **Exhibit B** shows that isolated cell populations of the present invention are positive for expression of the β2-M from at least 18.8 to at least 51.7 cell doublings. *See*, **Exhibit B**, col. 4, labeled "**Beta 2 Micro**", rows 1-5 corresponding to 18.8, 27.2, 35.2, 43.9, 51.7 population doublings (PD).

b) In contrast, in the SUMMARY OF THE INVENTION the Furcht patent teaches:

> In particular, the cell may be surface antigen negative for CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek, CD44, HLA-class 1 and 2-microglobulin...

Id. at col. 5, lines 58-67 (emphasis added).

In a section of the specification describing analysis of "Human MASCs" the Furcht patent teaches:

> Immunophenotypic analysis by FACS of human MASCs obtained after 22-25 cell doublings showed that cells do not express CD31, CD34, CD36, CD38, CD45, CD50, CD62E and -P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek; and express low levels of CD44, HLA-class I, and β2-microglobulin, but express CD10, CD13, CD49b, CD49e, CDw90, Flk1 (N>10).
>
> Once cells undergo >40 doublings in cultures re-seeded at $2x10^3/cm^2$, the phenotype becomes more homogenous and no cell expressed HLA-class-I or CD44 (n=6). When cells were grown at higher confluence, they expressed high levels of Muc18, CD44, HLA-class I and β2-microglobulin, which is similar to the phenotype described for MSC (N=8) (Pittenger, Science (1999) 284: 143-147). [**FN**[12]]

Id. at col. 16, lines 42-55 (emphasis added).

5. *CONCLUSION*

The phenotype of cells of the present invention differ substantially from the phenotype described for the cells in the Furcht patent. In particular, the cells of the present invention differ in the non-expression of CD10 and the consistently positive expression of CD44, HLA Class-1, and β 2-Microglobulin (from low to high numbers of cell doublings). In contrast, the cells in the Furcht patent show positive expression of CD10 and inconsistent expression of CD44, HLA Class-1, and β 2-Microglobulin (and the inconsistent capacity to propagate to high numbers of cell doublings). Thus, it is clear that the isolated cell populations of the present invention and the isolated cell populations described in the Furcht patent are different cell populations.

[12] In contrast to the cells described in the Furcht patent, the isolated cells of the present invention consistently express β2-M, even when cultured at low density. In evidence, the cells analyzed by FACs in Exhibit B were cultured at low density (*i.e.*, seeded at 30 cells/cm^2) and FACs analysis was performed on cells harvested from sub-confluent populations.

C. THE CONCLUSIONS ASSERTED IN DEANS WERE BASED ON AN EXPERIMENTALLY FLAWED APPROACH TO THE QUESTIONS ASKED

1. INTRODUCTION

The methods used to isolate bone marrow-derived cells in Deans were not the same methods described in the Furcht application. Instead, the isolation methods used in Deans appear to be a mixture of some of the methods described in Applicant's present application combined with some procedures described in the Furcht patent. Hence, given this hybrid approach, it would not be surprising if the cell populations described in Deans shared some attributes in common with the cells described and claimed in the present application. Most importantly, however, Deans did not test the isolated cell populations described in Furcht *et al* under the growth conditions described in Applicants present application (as Deans claims to have done) because the cell populations isolated in Deans were not the same as the isolated cell populations described in the Furcht patent.

2. METHODS TAUGHT IN THE FURCHT PATENT

The Furcht patent teaches isolating cells by depleting the bone marrow mononuclear cells of CD45+/glycophorin A+ cells through negative selection, plating the cells on matrix (*e.g.*, fibronectin) coated plates, and the necessity of culturing the cells in media supplemented with various growth factors. For example, the Furcht patent teaches:

> The present invention provides a method for isolating multipotent adult stem cells (MASC). The method involves depleting bone marrow mononuclear cells of $CD45^+$ glycophorin A^+ cells, recovering $CD45^-$ glycophorin A^- cells, plating the recovered $CD45^-$ glycophorin A^- cells onto a matrix coating, and culturing the plated cells in media supplemented with growth factors. The step of depleting may involved negative selection using monoclonal or polyclonal antibodies. The growth factors may be chosen from PDGF-BB, EGF, IGF, and LIF. The last step may further involve culturing in media supplemented with dexamethasone, linoleic acid, and/or ascorbic acid.

See, Furcht patent, col. 7, lines 1-12. *See also*, col. 44, line 51 to col. 45, line 35 (emphasis added).

The Furcht patent also teaches that, for human cells, the $CD45^-$/glycophorin A^- negative selection procedure may be performed immediately following Ficoll Hypaque separation, or after culturing on a fibronectin coated surface for 2-4 weeks, subsequently followed by negative selection. *See*, Furcht patent, col. 14, line 67 to col. 15, line 8. In contrast to human cells, the Furcht patent teaches that when murine marrow mononuclear are immediately depleted of $CD45^-$/glycophorin A^-, the cells do not survive and, therefore, these cells must be cultured for 14 days prior to negative selection. *See*, Furcht patent, col. 16, lines 18-25. The Furcht patent also teaches the *necessity* of culturing the isolated cells, whether murine or human, in the presence of supplemental growth factors; in particular, platelet derived growth factor-BB (PDGF-BB) and epidermal growth factor (EGF). *See e.g.*, Furcht patent: col. 15, lines 27-37; col. 16, lines 25-27; and, col. 45, lines 65-67.

3. METHODS DESCRIBED IN DEANS

a) Introduction

The bone marrow-derived cells analyzed in Deans were not isolated using the same procedures described for isolating a population of bone marrow-derived cells in the Furcht patent.

b) Deans Methods Did Not Use Immunoselection

In contrast to the Furcht patent, Deans is completely silent with respect to use of an immunoselection step during the cell isolation procedure. Therefore, it appears that an immunoselection step was omitted from the isolation procedures used in Deans. Despite this omission, Deans alleges that the "hBMC" or "BMC" cells (which is reportedly "an Athersys reference to cells obtained from human bone marrow culture"), were "*prepared in the same way* as the 'MASC' cells described in the Furcht PCT and...Furcht provisional applications..." *See*, Deans, page 4, item 8; *see also*, page 6, item 10 (emphasis added).

However, what it means to be "prepared in the same way" as described in the Furcht patent applications is also not apparent because the specification teaches multiple ways in which to prepare marrow-derived mononuclear cells (for example, such as already noted above). One step that is common, however, to the several cell isolation procedures taught in the Furcht patent is an immunoselection step involving either negative immunoselection for $CD45^-$ /glycophorin A^- cells, or positive immunoselection for leukemia inhibitory factor (LIF) $receptor^+$ cells. *See e.g.*, Furcht patent, col. 15, lines 1-12. Despite the stated importance of the immunoselection step [**FN**[13]], Deans never describes any such step being performed. In contrast, many other steps that would occur before and after this part of the isolation procedure are described in detail. For example, Deans describes the isolation of marrow mononuclear cells first by performing Ficoll density centrifugation, followed by resuspension in "MAPC" media (including a description of the formulation of this media), followed by seeding at 500,000 cells/cm^2 in fibronectin coated culture flasks. *See*, Deans, page 6, item 10; *see also*, page 4, item 9, a). Hence, it appears that the critical immunoselection procedure described in the Furcht patent was never performed on the bone marrow cells isolated as described in Deans. Thus, in Deans the bone marrow-derived cells were isolated using methods that did not recapitulate the methods described in the Furcht patent. Instead, Deans appears to have used a mixture of some of the procedures described in the Applicant's present application (for example, where mononuclear cells are isolated by performing density gradient centrifugation, followed directly by plating at low density in cell culture flasks without subjecting the cells to an immunoselection step) along with procedures described in the Furcht patent (such as growing the cells on fibronectin coated plates in the presence of dexamethasone (a potent synthetic hormone) along with supplemental PDGF-BB and EGF).

c) The Furcht Patent Teaches The Importance of Immunoselection

The crucial difference in cell types obtained when omitting immunoselection from the isolation procedure (as was done in Deans) compared to use of immunoselection (as is taught

[13] *See*, next section C, 3, c).

in the Furcht patent) is even more apparent when one considers that the Furcht patent specifically teaches the adverse consequences of omitting the immunoselection step. In this regard, the Furcht patent teaches:

> Sorted cells were plated in MASC culture with 10 ng/mL EGF, PDGF-BB and LIF. The frequency with which MASC started growing was 30-fold higher in $CD45^-$ $GlyA^-$ $LIF\text{-}R^+$ cells and 3 fold higher in $CD45^-$ $GlyA^-$ $CD44^-$ cells than in *unsorted* $CD45^-$ $GlyA^-$ cells.

See, Furcht patent, col. 15, lines 53-57 (emphasis added).

D. THOSE OF ORDINARY SKILL IN THE ART UNDERSTAND THAT CELL POPULATIONS WITH DIFFERENT GROWTH FACTOR REQUIREMENTS ARE NOT "ESSENTIALLY THE SAME" CELLS.

1. INTRODUCTION

Deans concludes that the cells described in Applicant's present application and "the cells prepared by the method of the Furcht '757 application [are] essentially the same". *See*, Deans, page 8, item 11; *see also*, item 13. In contrast to this assertion, however, the different growth factor requirements of these two cell populations demonstrates that these cell populations are *not* essentially the same.

2. CELLS ISOLATED IN THE FURCHT PATENT REQUIRE SUPPLEMENTAL GROWTH FACTORS

The requirement for supplemental growth factors demonstrates that the cells isolated in the Furcht patent are not the same as the isolated cells described in the present application. As discussed above, the Furcht patent teaches that the isolated bone marrow cells require supplemental growth factors. For example, the Furcht patent teaches:

> The present invention provides a method for isolating multipotent adult stem cells (MASC). The method involves depleting bone marrow mononuclear

> cells of $CD45^+$ glycophorin A^+ cells, recovering $CD45^-$ glycophorin A^- cells, plating the recovered $CD45^-$ glycophorin A^- cells onto a matrix coating, and culturing the plated cells in media supplemented with growth factors...The growth factors may be chosen from PDGF-BB, EGF, IGF, and LIF.

See, Furcht patent, col. 7, lines 1-10 (emphasis added).

> Cells are maintained in Dulbecco Minimal Essential Medium (DMEM) or other appropriate cell culture medium, supplemented with 1-50 ng/ml (preferably about 5-15 ng/ml) platelet-derived growth factor-BB (PDGF-BB), 1-50 ng/ml (preferably about 5-15 ng/ml) epidermal growth factor (EGF), 1-50 ng/ml (preferably about 5-15 ng/ml) insulin-like growth factor (IGF), or 100-10,000 IU (preferably about 1,000 IU) LIF, with 10^{-10} to 10^{-8} M dexamethasone or other appropriate steroid, 2-10μg/ml linoleic acid, and 0.05 0.15 μM ascorbic acid.

Id. at col. 15, lines 27-37 (emphasis added).

> Moreover, for isolated human cells the Furcht patent teaches:
>
> When cells were plated on collagen-type-I or laminin in stead of fibronectin, they expressed CD44 and HLA-DR, and could not be expanded beyond 30 cell doublings. When EGF or PDGF were omitted cells did not proliferate and died, while increased concentrations of these cytokines allowed initial growth of MASC but caused loss of proliferation beyond 20-30 cell doublings."

Id. at Example 1, col. 45, line 63 to col. 46, line 4.

> Likewise, for isolated murine cells the Furcht patent teaches:
>
> When cultured with PDGF-BB and EFG alone, cell doubling was slow (>6 days) and cultures could not be maintained beyond 10 cell doublings.

Id. at col. 16, lines 25-27.

3. CELLS ISOLATED IN THE PRESENT APPLICATION DO NOT REQUIRE SUPPLEMENTAL GROWTH FACTORS

In contrast to the Furcht patent, the isolated bone marrow-derived cells of the present invention do not require supplemental growth factors to grow and maintain proliferation through numerous population doublings. For example, the present specification teaches:

> Standard media preparations can be used to culture the bone marrow cells. For example, the media can be minimum essential medium-alpha modification supplemented with 4 mM L-glutamine and 0 to 10% lot selected fetal bovine serum (FSB), preferably about 10% FSB.

See, U.S. Pat. Pub. No. 20030059414, para. [0054].

> The resulting mononuclear cell pellet was resuspended in complete medium and centrifuged (10 minutes at 500xg). Complete media is Minimal Essential Medium-alpha (Gibco BRL, Rockville, Md.) supplemented with 4 mM glutamine and 10% sera-lot selected fetal bovine serum (FBS, Gibco BRL, Rockville, Md.).

Id. at para. [0115].

> ...adherent cells re-fed with fresh complete medium.

Id. at para. [0116].

> An aliquot of cells was removed from the Master Cell Bank and cultured at a density of 30 cells/cm^2 in 500 cm^2 tissue culture-treated plates (Corning) in complete medium...

Id. at para. [0127].

4. DIFFERENT CELL TYPES REQUIRE DIFFERENT GROWTH FACTORS IN ORDER TO DIVIDE

At least as early as 1989, it was well known in the art that different cell types require different growth factors in order to divide. In evidence, **Exhibit D** is submitted herein with a section from a leading textbook in cell biology, *Molecular Biology of The Cell, Second Edition* (Bruce Alberts *et al.*, Garland Publishing, Inc. (1989), Chapter 13 (Cell Growth and Division)), titled with exactly this heading:

> **Different Cell Types Require Different Growth Factors in Order to Divide**
>
> The conditions that must be satisfied before a cell will grow and divide are considerably more complex for an animal cell than for a yeast... Painstaking analyses have revealed that the essential components of serum are highly specific proteins, mostly present in very low concentrations... Different types of cells require different sets of these proteins. Some of the proteins in serum are directly and specifically involved in stimulating cell division and are called growth factors. One example is platelet-derived growth factor, or PDGF... The cells that respond to PDGF have specific receptors for PDGF - and for certain other growth factors - in their plasma membrane. Other cell types have other sets of receptors, mediating responses to other sets of growth factors...

5. CONCLUSION

The Furcht patent demonstrates the necessity of supplemental growth factors (*e.g.*, PDGF-BB, EGF, insulin) for maintaining cell proliferation of the bone marrow-derived cells described therein. In contrast, the bone marrow-derived cells of the present invention are able to grow without such supplements. Thus, based on their substantially different growth factor requirements, it would be readily apparent to those of ordinary skill in the art that the differently isolated cell populations in the Furcht patent versus the present application cannot be "essentially the same" and are, in fact, substantially different.

F. AFFIRMATION

I further declare that the above statements made of my own knowledge are true and the above statements based on information and belief obtained from the references and documents discussed are believed to be true. Additionally, I declare that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Title 18 United States Code Section 1001, and that willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Respectfully submitted,

Gene C. Kopen

Date: 17-MAY-07

EXHIBIT A

CURRICULUM VITAE

Name: Gene C. Kopen, Ph.D.

Address: 609 Argyle Rd.
Wynnewood, PA 19096
Home: 610-896-9001

Personal: Married, 3 children

Work Address: Neuronyx, Inc.
1 Great Valley Parkway, Suite 20
Malvern, PA 19355
Phone (610) 240-4157
Email: gkopen@neuronyx.com

RESEARCH AND RELATED PROFESSIONAL EXPERIENCE

I joined Neuronyx in June, 2000 as the 6th employee and was made responsible for recruiting and training R&D and animal facility staff, developing and implementing methodologies for large scale expansion of human stem cells, developing a framework for product development and testing, initiating collaborations with thought leaders in the field of neurodegeneration, and establishing an in-house pre-clinical program in spinal cord injury. By 2003 the company had approximately 51 employees, a GLP-compliant animal facility, cell manufacturing capability, and additional departments of Quality Control, Quality Assurance, and Clinical and Regulatory.

2003-Present **Sr. Scientist, Neuronyx, Inc.**

Current Responsibilities:

- Developing new clinical program opportunities leveraging platform technology
- Developing strategic R&D objectives in support of operating plan
- Managing milestone driven projects leading to value-adding product-development and process-development opportunities
- Managing Intellectual property portfolio
- Developing new technologies for next generation of biopharmaceuticals
- Directing core projects aimed at Cell Product characterization
- Managing IND-enabling pre-clinical research conducted through key academic collaborations and contract research organizations

Accomplishments:

- Part of cardiovascular IND team leading to compilation, submission and clearance of Phase I clinical Trial in Sub-Acute Myocardial Infarction (NCT00361855)
- Part of Manufacturing team leading to GMP processes for Manufacture of clinical-grade human ABM-SC, NX-CP015
- Portfolio of pharmacokinetic and toxicology studies supporting Stroke clinical development. Efficacy studies conducted through sponsored research lead to Grant from Department of Health and Human Services, State of Illinois.
- Development of bioassay systems and testing used to establish technical specifications for Manufacturing and Quality Control

2000-2003 Scientist, Neuronyx, Inc.

Responsibilities included:

- Development of technologies for isolation and *ex vivo* expansion of various human stem cells and somatic cells
- Implemented animal model of spinal cord injury (SCI) to study therapeutic potential of cell prototypes; trained and managed LAF staff, developed protocols for surgical & LAF procedures, GLP pre-clinical studies, etc.
- Managed many projects aimed at cell differentiation, characterization, and manufacture, as well as, pre-clinical development of cell prototypes
- Recruited and trained key positions for R&D and early phase-Manufacturing
- Managed IND-enabling pre-clinical research conducted through key academic collaborations and contract labs

Accomplishments:

- Developed *ex vivo* expansion technologies for human stem cells; this early work was quickly enabled for scale-up by other members of the team, leading to our current GMP manufacturing capacity of approximately 5×10^9 clinical doses of human ABM-SC from a single donor
- Assembled a pre-clinical SCI team in tandem with an academic collaboration that lead to 11+ pre-clinical studies supporting path to IND

1999-2000 Post-Doctoral Fellow, Center for Gene Therapy, MCP Hahnemann University, Philadelphia, PA.

Selected Scientific Projects (much of this work was initiated in parallel with my dissertation research):

- Development of new methods for isolation and *ex vivo* expansion of bone marrow stromal cells (MSC)
- Characterization of murine and human-derived MSC by flow cytometry, *in vitro* differentiation, *in vivo* pharmacology, as ell as various molecular techniques
- Identification of therapeutic potential of MSC in animal model of storage disease
- Development of *in vitro* differentiation assays aimed at mesenchymal-to-epithelial differentiation of MSC

1995-1999 Graduate Student. Doctoral Program Molecular Pathobiology, MCP Hahnemann University, Philadelphia, PA.

Mentor: Darwin Prockop M.D, Ph.D. Center for Gene Therapy

Thesis: Murine Bone Marrow Stromal Cells: Characterization, Isolation, and Differentiation

Other: Vice President, Student Government, Graduate Admissions committee member

1993-1995 Clinical Laboratory Manager, Department of Pathology, Medical College of Pennsylvania, Philadelphia, PA

Responsibilities included:

- Supervision of all facets of diagnostic Immunohistochemistry (IHC) and assay development for 10 + area hospitals and research institutions
- Conducted Image analyses of breast cancers for ER/PR
- DNA ploidy by Flow Cytometry
- Conducted diagnostic Immunofluorescence on renal and skin bx
- Stat cryosectioning and histology on surgically resected bx
- Training cytology students and local researchers on methods of IHC
- Developed and optimized techniques for diagnostic detection of various tissue antigens
- Patient billing and lab QC
- Other duties included examination, processing, and dictation of gross exam on surgical specimens. Autopsy duties, when needed, included organ removal and processing for histopathology

1992-1995 Research Associate, (part-time) **Department of Pathology, Medical College of Pennsylvania, Philadelphia, PA.** Laboratory of James England, MD., Ph.D.

Lab focus was study of oncogenic events responsible for mesothelioma formation and
V-SRC mediated tumor formation and metastasis.

1992 Student Volunteer, Labor and Delivery, Ob/Gyn, Hospital of the University of Pennsylvania, Phila., PA. (Part-time, Summer)

Volunteer training program. Program objective to give pre-doc students clinical exposure.

1990-1992 Research Associate, Department of Pathology, Medical College of Pennsylvania, Philadelphia, PA. (Full-time) Laboratory of James England, MD., Ph.D.

Lab focus was study of immunologic mechanisms controlling *V-SRC* induced tumor cell growth and metastasis. This work was conducting in collaboration with Michael Halpern, Ph.D, of the Wistar Institute, Philadelphia, PA.

1990 Psychiatric Technician, Eastern Pennsylvania Psychiatric Institute (Full-time, Summer)

Principle responsibilities included, conducting patient history & physical assessment for new admissions. Patient support & monitoring, maintaining patient compliance to therapeutic regiments.

1988 Sales & Marketing, Eastern Telephone Systems, Fort Washington, PA. (Full-Time)

Sales and marketing of commercial services, client services.

1987 Internship, Kidder, Peabody, & Co, Tampa, Fl. Brokerage and Client Services.

EDUCATION AND TRAINING

Univ. of South Florida, Tampa, FL	**B.A. Social & Behavioral Sciences, 1987**
Univ. of Pennsylvania, Phila., PA **1991-**	**Post-Baccalaureate Program, Pre-Health, 1994**
Drexel College of Medicine, Phila., PA	**Ph.D. Molecular Pathobiology, 1999**

PUBLICATIONS

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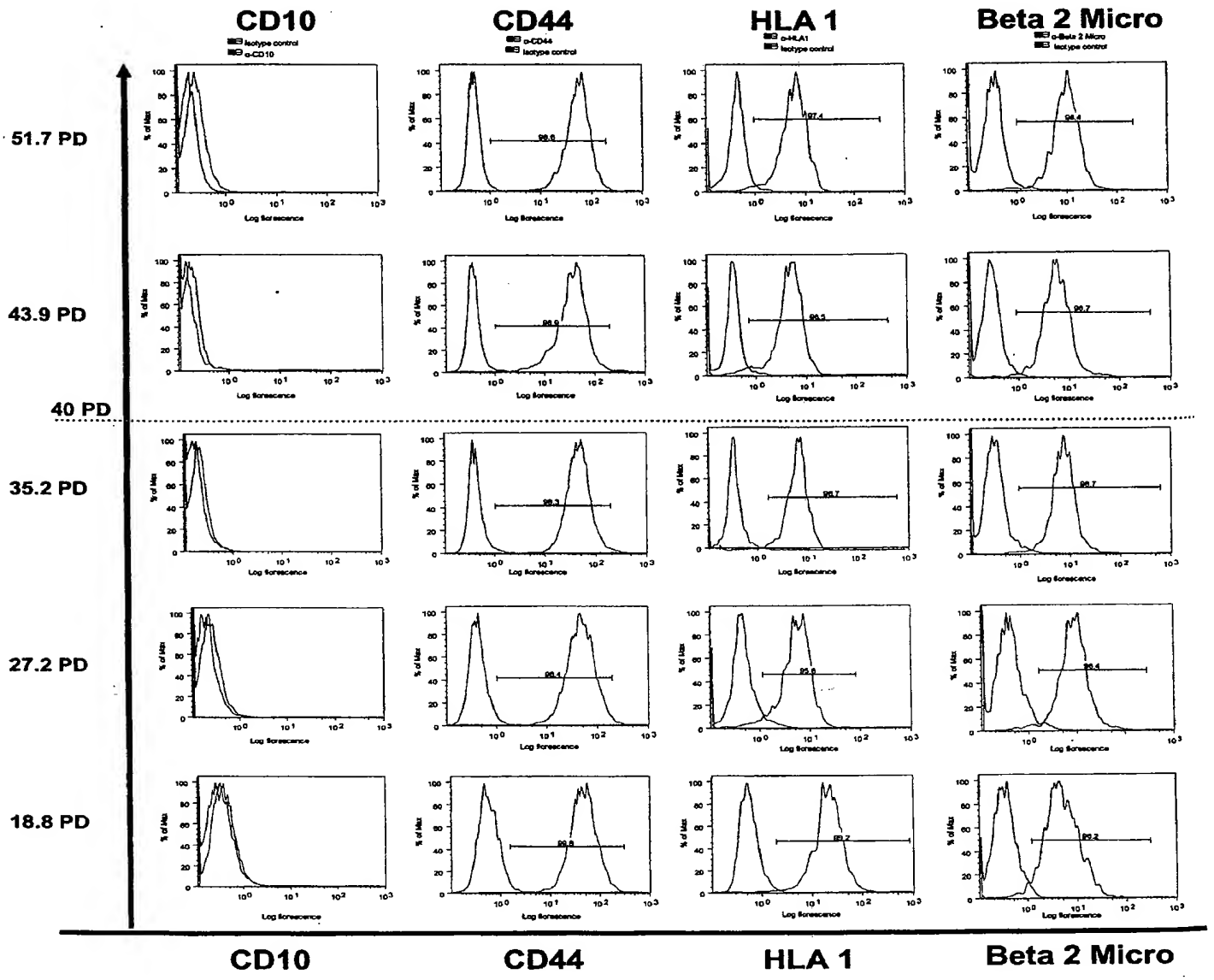
EXHIBIT B



Description in Furcht *et al.*

CD10

"In particular, the cell may be surface antigen negative for CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek, CD44, HLA-class 1 and 2-microglobulin [sic] and is positive for CD 10..." *See*, Summary of the Invention, col. 5, ll. 63-67. (*See also*: col. 16, ll. 40-48; col. 45, ll. 43-51; col. 16, l. 62 to col. 17, l. 7; and, col. 53, ll. 42-56.)

CD44

"The present invention provides an isolated multipotent mammalian stem cell that is surface antigen negative for CD44, CD45, and HLA Class I and II." *See*, Summary of the Invention, col. 5, ll. 58-60. (*See also*: col. 16, ll. 42-55; col. 18, ll. 26-28; Example 1, col. 45, ll. 38-62.)

HLA-CLASS-1

"The present invention provides an isolated multipotent mammalian stem cell that is surface antigen negative for CD44, CD45, and HLA Class I and II. The cell may also be surface antigen negative for CD34, Muc18, Stro-1, HLA-class-I and may be positive for oct3/4 mRNA, and may be positive for hTRT mRNA. In particular, the cell may be surface antigen negative for CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek, CD44, HLA-class 1 and 2-microglobulin [sic] and is positive for CD 10..." *See*, Summary of the Invention, col. 5, ll. 58-67. (*See also*: col. 16, ll. 42-55; and, Example 1, col. 45, ll. 38-62.)

β-2 Microglobulin

"In particular, the cell may be surface antigen negative for CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek, CD44, HLA-class 1 and 2-microglobulin [sic] and is positive for CD 10..." *See*, Summary of the Invention, col. 5, ll. 63-67. (*See also*: col. 16, ll. 42-55.)

40 Population Doublings

"Immunophenotypic analysis by FACS of human MASCs obtained after 22-25 cell doublings showed that cells do not express CD31, CD34, CD36, CD38, CD45, CD50, CD62E and -P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek; and express low levels of CD44, HLA-class I, and beta2-microglobulin, but express CD10, CD13, CD49b, CD49e, CDw90, Flk1 (N>10). Once cells undergo >40 doublings in cultures re-seeded at $2x10^3/cm^2$, the phenotype becomes more homogenous and no cell expressed HLA-class-I or CD44 (n=6)." (Col. 16, ll. 42-55.)

EXHIBIT C

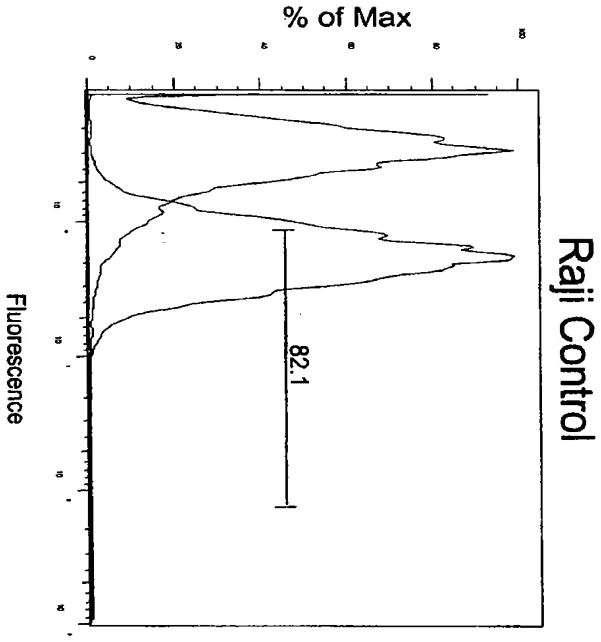


EXHIBIT D

MOLECULAR BIOLOGY OF THE CELL

SECOND EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

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The Cycle Times of Proliferating Cells Seem to Depend on a Probabilistic Event[18]

Individual cells that are freely dividing in culture can be continuously observed by time-lapse cinematography. Such studies reveal that even cells that are genetically identical have widely variable cycle times (Figure 13–24). A quantitative analysis suggests that the time from one division to the next includes a randomly variable component, with the random variation occurring chiefly in the duration of the G_1 phase. It seems that, as cells approach the restriction point in G_1 (Figure 13–25), they must "wait" to embark on the rest of the cycle and that each waiting cell has a roughly constant probability per unit time of passing R. Thus, like a population of atoms undergoing radioactive decay, if half of the cells pass R within the first 3 hours, one-half of the remaining cells will pass R within the next 3 hours, one-half of the remainder after that will pass R within the next 3 hours, and so on. A possible mechanism that might explain such behavior was suggested previously when we discussed the production of the S-phase activator (see p. 733). But regardless of its cause, the random variability that is observed in the length of the cell-division cycle means that initially synchronous cell populations will drift out of synchrony within a few cycles. This is awkward for cell biologists but may be advantageous for the multicellular organism—otherwise, large clones of cells might pass through mitosis simultaneously; and since cells tend to round up and lose their attachments during mitosis, this could seriously disrupt the tissue they belong to.

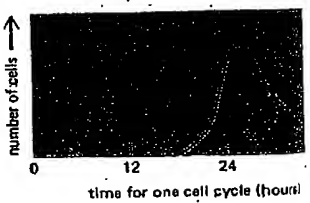

Figure 13–24 The variation in c cycle times typically observed for homogeneous population of cells growing in tissue culture. Such d obtained by observing individual the microscope and directly mea the time between their successive divisions.

13-15 13-16 Different Cell Types Require Different Growth Factors in Order to Divide[19,20]

The conditions that must be satisfied before a cell will grow and divide are considerably more complex for an animal cell than for a yeast. If vertebrate cells in a standard artificial culture medium are completely deprived of serum, they normally will not pass the restriction point, even though all the obvious nutrients are present; and they will halt their growth as well as their progress through the chromosome cycle. Painstaking analyses have revealed that the essential components of serum are highly specific proteins, mostly present in very low concentrations (on the order of 10^{-9} to 10^{-11} M). Different types of cells require different sets of these proteins. Some of the proteins in serum are directly and specifically involved in stimulating cell division and are called **growth factors.** One example is **platelet-derived growth factor,** or **PDGF.** The path to its isolation began with the observation that cultured fibroblasts proliferate when provided with serum but not when provided with plasma—the liquid component of blood, prepared by removing the blood cells without allowing clotting to occur. When blood clots, the platelets (see p. 974) are triggered to release the contents of their secretory vesicles; and among the products released (along with factors that promote clotting) is PDGF. It is mainly the PDGF in the serum that enables fibroblasts in culture to divide. PDGF presumably has the same effect in the body, where it is thought to stimulate connective tissue cells and smooth muscle cells to divide at the site of a wound to help repair the damage (Figure 13–26). The cells that respond to PDGF have specific receptors for PDGF—and for certain other growth factors—in their plasma membrane. Other cell types have other sets of receptors, mediating responses to other sets of growth factors (Table 13–1), several of which are also present in serum.

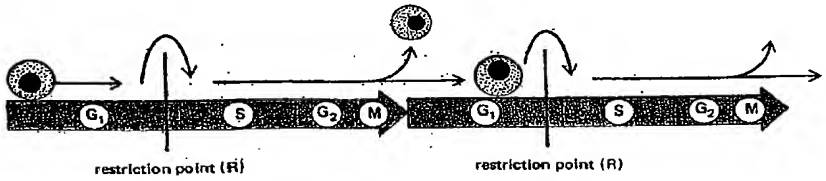

Figure 13–25 Cartoon indicati rapidly dividing mammalian cell culture are delayed, for a variabl of time, at a point late in G_1, wh be the same as the restriction p at which they will halt indefinit protein synthesis is inhibited. Fi continuous observation of cells microscope, it seems that genet identical cells, including the tw daughter cells that result from a division, are often delayed for v different amounts of time before passing R, suggesting that the d due to some random process (s

JOURNAL OF CELLULAR PHYSIOLOGY 187:345–355 (2001)

Cultivation of Rat Marrow-Derived Mesenchymal Stem Cells in Reduced Oxygen Tension: Effects on In Vitro and In Vivo Osteochondrogenesis

DONALD P. LENNON,[1] JOHN M. EDMISON,[1,2] AND ARNOLD I. CAPLAN[1]*
[1]*Skeletal Research Center, Case Western Reserve University, Cleveland, Ohio*
[2]*School of Medicine, Case Western Reserve University, Cleveland, Ohio*

Rat mesenchymal stem cells (rMSCs) represent a small portion of the cells in the stromal compartment of bone marrow and have the potential to differentiate into bone, cartilage, fat, and fibrous tissue. These mesenchymal progenitor cells were maintained as primary isolates and as subcultured cells in separate closed modular incubator chambers purged with either 95% air and 5% CO_2 (20% or control oxygen) or 5% oxygen, 5% CO_2, and 90% nitrogen (5% or low oxygen). At first passage, some cells from each oxygen condition were loaded into porous ceramic vehicles and implanted into syngeneic host animals in an in vivo assay for osteochondrogenesis. The remaining cells were continued in vitro in the same oxygen tension as for primary culture or were switched to the alternate condition. The first passage cells were examined for in vitro osteogenesis with assays involving the quantification of alkaline phosphatase activity and calcium and DNA content as well as by von Kossa staining to detect mineralization. Cultures maintained in low oxygen had a greater number of colonies as primary isolates and proliferated more rapidly throughout their time in vitro, as indicated by hemacytometer counts at the end of primary culture and increased DNA values for first passage cells. Moreover, rMSCs cultivated in 5% oxygen produced more bone than cells cultured in 20% oxygen when harvested and loaded into porous ceramic cubes and implanted into syngeneic host animals. Finally, markers for osteogenesis, including alkaline phosphatase activity, calcium content, and von Kossa staining, were elevated in cultures which had been in low oxygen throughout their cultivation time. Expression of these markers was usually increased above basal levels when cells were switched from control to low oxygen at first passage and decreased for cells switched from low to control oxygen. We conclude that rMSCs in culture function optimally in an atmosphere of reduced oxygen that more closely approximates documented in vivo oxygen tension. J. Cell. Physiol. 187: 345–355, 2001. © 2001 Wiley-Liss, Inc.

The remarkable growth in the use of cell culture as a research tool which followed the pioneering work of Ross Harrison, Alexis Carrel, and others in the early part of the 20th century (Russell, 1969; Harvey, 1975) was accompanied by the equally rapid development of various undefined and defined culture media. Closely linked with some of the changes in culture media were adjustments to the atmosphere in which cells were maintained. Most of these adjustments involved variations in the concentration of carbon dioxide. With a few exceptions, such as Leibovitz's L-15 (Leibovitz, 1963) and media based on Hanks' balanced salt solution (Freshney, 1987), most media rely on the use of 5% CO_2 in conjunction with sodium bicarbonate or other buffers to maintain proper pH (Freshney, 1987). The slight reduction in oxygen concentration coincident with the use of 5% CO_2 was not found to have any deleterious effects on cell function.

While a small number of investigators (Trowell, 1959; DeRidder and Mareel, 1978) found a requirement for higher than ambient oxygen concentrations in organ (but not cell) culture, others determined that some cells proliferate more rapidly in oxygen concentrations lower

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than 20%. Cooper et al. (1958) and Zwartouw and Westwood (1958) were among the first to document these results. An explanation for the phenomenon of increased cellular proliferation in reduced oxygen tension in vitro was articulated by Packer and Fuehr (1977) and expanded by others, including Rich and Kubanek (1982) and Rich (1986), and is briefly summarized as follows. The oxygen tension (or partial pressure) in most in vitro settings (~140 mm Hg) is considerably higher than that found in most mammalian and avian tissues. For example, the partial pressure of oxygen (pO_2) in arterial blood has been measured at 60–90 mm Hg by Grant and Smith (1963) and at 75–110 mm Hg by Tøndevold et al. (1979). These reports, and that of Kofoed et al. (1985), place the pO_2 of bone marrow in the 27–49 mm Hg range. These tensions correspond to an oxygen concentration of approximately 4–7%. While organisms have evolved sophisticated mechanisms, including the enzymes glutathione peroxidase, catalase, and superoxide dismutase, and use the antioxidants ascorbate and vitamin E to defend themselves against the toxic effects of free radicals derived from oxygen (Frank and Massaro, 1980; Halliwell, 1984), it is possible that these mechanisms are inadequate to protect cells when oxygen concentrations are unusually high. Thus, it is conceivable that many cells would function more normally in vitro at oxygen concentrations lower than 20%.

Indeed, investigators have reported that reduced oxygen tension enhances proliferation of many cell types, including bovine periosteal cells (Deren et al., 1990), bovine pericytes (Brighton et al., 1992), rat calvarial osteoblasts (Tuncay et al., 1994), chick embryonic chondrocytes (Nevo et al., 1988), fibroblasts from human (Taylor et al., 1974; Balin et al., 1984) and murine (Fisher, 1960) sources, and hematopoietic progenitor cells derived from human (Bradley et al., 1978; Magda et al., 1986; Koller et al., 1992a,b) and murine marrow or umbilical cord blood (Rich and Kubanek, 1982; Rich, 1986; Koller et al., 1992b). Moreover, in vitro cultivation in reduced oxygen affects other aspects of the physiology of some cells, as indicated by increased alkaline phosphatase expression in periosteal cells (Deren et al., 1990; Reilly et al., 1998) and pericytes (Brighton et al., 1992), enhanced responsiveness of erythropoietic cells to erythropoietin (Rich and Kubanek, 1982; Maeda et al., 1986; Rich, 1986), and increased stimulation of macrophage colony formation in response to M-CSF (Broxmeyer et al., 1990). Lastly, this laboratory showed that 5% oxygen is optimal for the cultivation and in vitro differentiation of embryonic chick limb bud mesenchymal cells (Caplan and Koutroupas, 1973).

In the current study, we investigated the effect of reduced (5%) oxygen in vitro on rat marrow-derived mesenchymal stem cells (rMSCs); this oxygen tension approximates that of bone marrow in vivo, as described above. These pluripotent cells, which reside in the stromal compartment of bone marrow, have been extensively characterized (Maniatopoulos et al., 1988; Leboy et al., 1991; Dennis et al., 1992; Lennon et al., 1995; Zhang et al., 1995; Cassiede et al., 1996; Hanada et al., 1997) and are capable, given the appropriate environmental cues, of progressing along one of a number of differentiation pathways, including those giving rise to bone (Maniatopoulos et al., 1988; Aubin et al., 1990; Leboy et al., 1991; Dennis et al., 1992), cartilage (Dennis and Caplan, 1993; Lennon et al., 1995; Mann et al., 1996), fat (Wakitani et al., 1995), or muscle (Wakitani et al., 1995).

Two different models have been used in our laboratory to investigate the differentiation of rMSCs along osteochondrogenic pathways. In the first, osteochondral differentiation is induced by the addition of dexamethasone to first passage rMSCs, while untreated control cultures remain in an undifferentiated state (Maniatopoulos et al., 1988; Aubin et al., 1990; Leboy et al., 1991; Cassiede et al., 1996; Hanada et al., 1997). In the second model, rMSCs maintained in vitro as primary cultures are trypsinized and loaded into porous ceramic vehicles. Bone and cartilage develop within these composites when they are implanted subcutaneously in syngeneic host animals (Dennis et al., 1992; Dennis and Caplan, 1993).

Our goal in the present study is to address the following questions. Does cultivating rMSCs in 5% oxygen affect their rate of proliferation? Are dexamethasone-treated rMSCs more likely to express osteoblastic characteristics when cultured in reduced oxygen? Are these results altered when the oxygen concentration is changed at first passage? Finally, are rMSCs maintained in vitro in 5% oxygen affected in their ability to produce bone or cartilage in porous ceramic cubes in vivo.

The data indicate that proliferation of rMSCs is increased in both primary culture and first passage in an atmosphere of reduced oxygen, that markers for osteogenic differentiation are elevated for cells in low oxygen in first passage, and that rMSCs maintained in 5% oxygen in primary culture are likely to produce more bone and cartilage when returned to an in vivo setting than are those cultivated in 20% oxygen.

MATERIALS AND METHODS

Cell culture

Rat mesenchymal stem cells: isolation and primary culture. Rat MSCs were isolated as previously described (Dennis et al., 1992; Lennon et al., 1995). Briefly, male F-344 rats, 6–12 weeks of age, were sacrificed by CO_2 asphyxiation according to Institutional Animal Care and Use Committee (IACUC) guidelines. The tibiae and femora were aseptically removed and adherent soft tissue was thoroughly debrided. After the epiphyses were removed, an 18-gauge needle attached to a 10-ml syringe was used to bore a small opening through the growth plate on the distal end of the femora and the proximal end of the tibiae. A 10-ml syringe fitted with a fresh 18-gauge needle was filled with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG; Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan UT) from a lot previously tested and found to support the preferential attachment, proliferation, and differentiation of rMSCs (Lennon et al., 1996). The needle was inserted into the opening previously prepared, and a small volume of medium was ejected to expel the bone marrow from the medullary canal. Marrow samples were collected and mechanically disrupted by passing them successively through 16-, 18-,

and 20-gauge needles. Disaggregated marrow was centrifuged at 500*g* in a Beckman TJ-6 centrifuge and resuspended in serum-supplemented medium; an aliquot of the cell suspension was combined with an equal volume of 4% (v/v) acetic acid to lyse red blood cells, and nucleated cell numbers were determined with a hemacytometer. Cells were seeded at 5×10^7/100-mm tissue culture dish.

Equal numbers of culture dishes were placed inside two air-tight modular incubator chambers (Billups-Rothenburg, Del Mar, CA). Humidity was supplemented by placing an open tissue culture dish containing 10–20 ml of sterile distilled water in the bottom of each chamber. Gas mixtures, consisting of 5% oxygen, 5% CO_2, and 90% nitrogen (referred to as low or 5% oxygen) for one chamber and 5% CO_2 and 95% air for the other (referred to as control or 20% oxygen), were allowed to flow into the chambers through one of the two ports, thus flushing ambient air from the chamber. The airflow was stopped after 15 min and both ports were immediately closed. The chambers were then placed inside a standard tissue culture incubator.

In order to ensure that pressure inside the chamber was comparable to that inside the incubator, gas was periodically vented from the chamber until the temperature inside the chamber equaled that inside the main incubator. The modular chambers were flushed with fresh gas daily, and excess gas was vented in the same manner. Cells were continued in control or low oxygen conditions for the duration of their time in primary culture. However, at first passage, half of the cultures generated for each of the two oxygen conditions were continued in the same atmosphere, while the other half were switched to the alternate condition. Culture medium was removed and replaced with fresh serum-supplemented medium after 3 days and every 3–4 days thereafter. In one experiment, the pH of medium collected from culture dishes at each medium change in primary culture was determined with a pH meter (Accumet model 10, Fisher Scientific, Pittsburgh, PA).

Cell subculture

When colonies of rMSCs increased in size, but prior to the time they became multilayered and the colonies came in contact with one another (usually on day 12 of primary culture), the cells were subcultured by treatment with 0.25% trypsin in 1 mM EDTA (Life Technologies, Grand Island, NY) for 5 min at 37°C. Trypsinization was arrested with the addition of ½ volume of calf serum (Hyclone Laboratories), and the resulting cell suspension was centrifuged at 500*g* for 5 min, resuspended in serum-free DMEM-LG, and counted with a hemacytometer. A portion of the cell suspension was reserved for an in vivo osteogenic assay, while the remainder was resuspended in serum-containing medium and seeded into 35-mm culture dishes at a density of 10^5 cells per dish for use in an assay for in vitro osteogenesis; both assays are described in detail below. A flow chart outlining the experimental design is illustrated in Figure 1.

In vitro osteogenesis

In order to assess the effect of oxygen concentration on in vitro osteogenesis, cultures of first passage rMSCs maintained in primary culture in 5% or 20% oxygen were each divided into two equal groups immediately after being subcultured (day 0). One group was continued in the same oxygen condition in which it was maintained in the previous passage, while the other was switched to the alternate oxygen condition. Thus, four groups of cultures were generated: those which were in either control or low oxygen throughout their time in culture (referred to as C-C and L-L respectively) and those switched from control to low (C-L) or from low to control (L-C) oxygen after being seeded into 35-mm dishes.

Medium was changed for all cultures on day 1 of first passage. Serum-containing DMEM-LG, further augmented with osteogenic supplements (OS), consisting of 100 nM dexamethasone (Sigma Chemical) and 80 μM ascorbic acid 2-phosphate (Wako Chemicals, Richmond, VA), was added to one half of the cultures in each of the four groups, while control medium (DMEM-LG supplemented with 10% FBS and 80 μM ascorbic acid-2-phosphate) was added to the remainder. On day 10, all culture media were further augmented with 10 mM β-glycerophosphate (BGP; Sigma Chemical). Cells were continued in control or OS medium for the remainder of their time in vitro. The goal of this part of the study was to determine whether low oxygen concentration in primary culture affects the commitment of cells to the osteogenic lineage, or whether the observed effects are on osteogenic expression and are limited primarily to the time of exposure to the respective oxygen tensions. The frequency of medium changes and of gas replenishment was the same as for primary culture. In vitro osteogenesis was evaluated quantitatively through assays for calcium content and alkaline phosphatase activity as described below.

Alkaline phosphatase assay

Alkaline phosphatase activity was determined for first passage rMSCs in control or OS medium on days 3, 6, 9, 12, and 16 of culture. Triplicate cultures from each experimental group were rinsed twice with Tyrode's balanced salt solution (Sigma Chemical), and 1 ml of a 1 mg/ml solution of alkaline phosphatase substrate (p-nitrophenyl phosphate; Sigma Chemical) in a buffer consisting of 50 mM glycine and 1 mM $MgCl_2 \cdot 6H_20$ (pH 10.5) was added per 35-mm dish. After 10 min the solution was removed and transferred to a tube containing an equal volume of 1 M NaOH. Appropriately diluted samples of the resulting solutions were transferred to a 96-well culture dish, and the absorbance was read at 405 nm on a model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA). A standard curve generated from a series of dilutions of *p*-nitrophenol (Sigma Chemical) was used to determine the concentration of the enzyme reaction product. After the alkaline phosphatase assay solution was removed, the cultures were rinsed twice with Tyrode's salt solution and fixed with 100% ethanol. After 15 min, the ethanol was removed and the plates were dried and stored at 4°C until used for quantification of DNA.

Calcium

On day 21, triplicate rMSC cultures were rinsed twice with Tyrode's salt solution and fixed with 1% (v/v)

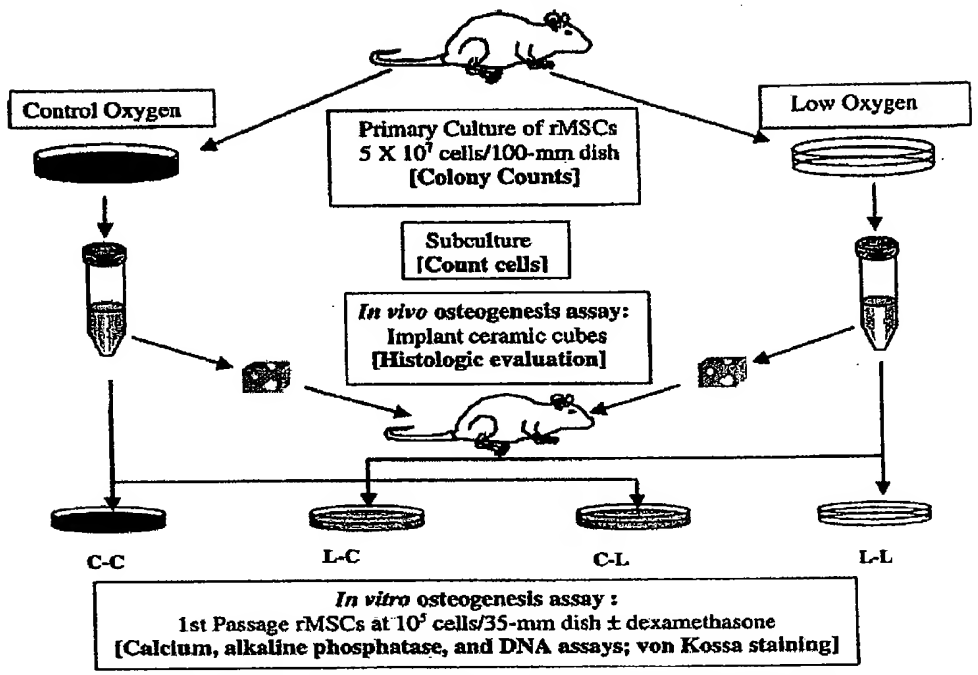

Fig. 1. Flow diagram of the experimental procedure. Rat mesenchymal stem cells (rMSCs) were seeded into 100-mm dishes and placed inside closed modular incubator chambers purged with 20% (control) or 5% (low) oxygen. In some cases, the number of cell colonies per culture was determined for primary cultures. At first passage, cell numbers were determined by hemacytometer count, and subcultured cells were tested for osteochondrogenesis with in vivo and in vitro assays. For the in vivo assay, rMSCs were loaded into porous ceramic cubes and implanted subcutaneously into syngeneic hosts; cubes were harvested after 3 or 6 weeks and evaluated for bone or cartilage. Remaining first passage cells were seeded into 35-mm dishes and continued in the same atmosphere or transferred to the alternate oxygen condition, thus generating four culture groups: C-C, C-L, L-L, and L-C (where C = control or 20% oxygen and L = low or 5% oxygen; the first and second letters indicate oxygen concentration in primary culture and first passage, respectively). On day 1 of first passage, each of these four groups was further divided into two subgroups, one of which was supplemented with ascorbic acid 2-phosphate (control medium) and the other with ascorbic acid 2-phosphate and 10^{-7} M dexamethasone (OS medium). First passage cultures were used in assays to quantify calcium content, alkaline phosphatase activity, and DNA and for von Kossa staining to detect mineralization.

glutaraldehyde in Tyrode's for 30 min. Following fixation, cultures were rinsed twice with distilled water, allowed to dry, and stored at 4°C. Calcium was extracted with 1 ml of 0.6 M HCl per dish. Dishes containing this solution were placed on a rotary shaker and maintained at a speed of 50 rpm overnight. Aliquots of the extract, diluted as needed, were added to wells of a 96-well culture dish (4 wells/sample); reagents from a commercial calcium assay kit (Sigma Chemical) were added to the wells, and the absorbance was read at 575 nm with a model 2550 microplate reader (Bio-Rad Laboratories). Calcium concentration was determined with a standard curve generated from a series of dilutions of $CaCl_2$.

DNA

DNA content was quantified by a modification (Hanada et al., 1997) of a technique described by Gillery et al. (1993). Triplicate cultures were rinsed twice with Tyrode's salt solution and fixed with 100% ethanol for 15 min, either after quantification of alkaline phosphatase activity (day 3 through 16) or independent of other assays (day 21). Dehydrated cultures were stored at 4°C until all were collected. All samples from each preparation were analyzed for DNA content at the same time. One ml of 40% (w/v) 3,5-diaminobenzoic acid (DABA, Sigma Chemical), twice clarified by adsorption to granular charcoal and then filtered through a 0.22-μm Nalgene filter unit (Nalgene Corp.), was added per 35-mm tissue culture dish. The dishes were incubated at 60°C for 45 min and the DABA-DNA reaction was stabilized by the addition of 2.5 ml of 3N HCl. The optical density of the resulting solution was determined with a spectrophotofluorometer at an absorbance of 420 nm and an emission of 490 nm. A standard curve generated from a series of dilutions of calf thymus DNA was used to determine the DNA concentration of the samples. Differences in DNA content are assumed to reflect differences in cell number.

von Kossa staining

The presence of mineralized deposits in first passage rMSC cultures was demonstrated with von Kossa

staining. On day 21, triplicate cultures of each experimental group were rinsed twice with Tyrode's salt solution, fixed with 1% glutaraldehyde (v/v) for 15 min, and rinsed three times with distilled water. One ml of 2% (w/v) silver nitrate (Sigma Chemical) was added per dish, and the cultures were placed in a dark environment for 10 min. Cultures were then rinsed three times with distilled water and exposed to bright light (while covered with water) for 15 min. Culture dishes were rinsed again with water and then dehydrated with 100% ethanol.

Colony counts

For some rMSC preparations, colony numbers were determined between days 7 and 12 of primary culture (when colonies were large but not overlapping). Cultures maintained in either control or low oxygen were rinsed twice with Tyrode's salt solution, fixed for 15 min with 1% (v/v) glutaraldehyde, rinsed twice with distilled water, and air dried. Fixed cultures were stained with 4 ml of 0.1% (w/v) crystal violet in distilled water for 30 min, rinsed three times with distilled water, and air dried. Inverted culture dishes were examined on a dissecting microscope at low magnification (7X), and the number of colonies (of more than 50 cells) was determined. All cultures were evaluated by the same individual, who was unaware of the identity of the cultures.

In vivo osteogenic assay

Implantation of MSC-loaded ceramic cubes into syngeneic or immunocompromised host animals as an in vivo assay for the osteochondrogenic potential of these cells has been described previously (Dennis et al., 1992, Dennis and Caplan, 1993). Briefly, blocks of porous ceramic (mean pore size of 200 μm), consisting of 60% tricalcium phosphate and 40% hydroxyapatite, were cut into cubes measuring 3 mm per side. The ceramic cubes were washed with water to remove ceramic dust, dried under a heating lamp, and sterilized in an autoclave. To improve cell attachment, ceramic cubes were combined with a 100 μg/ml solution of human fibronectin (Collaborative Biomedical, Bedford, MA) in Tyrode's salt solution in a 12 × 75-mm sterile, capped polystyrene tube (Becton Dickinson). A 20-gauge needle attached to a 30-ml syringe was inserted through the cap of the tube, and the syringe plunger was retracted to evacuate air from the tube, thus generating a partial vacuum and permitting the fibronectin solution to enter the pores of the cubes. Cubes remained in the fibronectin solution for 2 h at room temperature and were then air dried overnight in a laminar flow hood.

Rat MSCs trypsinized after primary culture in control or low oxygen (as described above) were resuspended at 5×10^6 cells/ml in serum-free DMEM-LG and transferred to separate 12 × 75-mm sterile tubes. Fibronectin-coated ceramic cubes were added and air was withdrawn with a 20-ml syringe as for fibronectin coating. Cell-loaded cubes were incubated at 37°C for 2 h and then implanted subcutaneously into pockets created by blunt dissection on the dorsal surface of syngeneic rats anesthetized with sodium pentobarbital (90 mg/kg; Veterinary Laboratories, Inc., Lenexa, KS).

Host animals were sacrificed after 3 or 6 weeks by CO_2 asphyxiation. Ceramic cubes were removed, fixed with 10% buffered formalin phosphate (Fisher Scientific), decalcified with RDO (Apex Engineering, Plainfield, IL), embedded in Tissue Prep 2 (Fisher Scientific), and cut into sections 5 μm in thickness. Each 10^{th} and 11^{th} section of a cube was collected and placed onto alternate glass slides; paired slides were stained with either toluidine blue or Mallory-Heidenhain. Stained sections were examined for the presence of bone or cartilage by brightfield microscopy with an Olympus IMT-2 inverted microscope. The total number of pores for each ceramic cube and the number of those pores which contained bone or cartilage were determined for each section, and the percentage of bone- or cartilage-positive pores was calculated. Histologic sections were evaluated on a double blind basis, and all were graded by the same individual (DPL).

Statistical analysis

Statistical analysis was conducted with Sigma Stat software (SPSS Inc., Chicago, IL). Student's paired *t*-test was used to analyze cell yields at the end of primary culture and ceramic cube data from the in vivo osteogenic assay. Colony counts for cells in low and control oxygen were compared with Student's *t*-test. DNA, alkaline phosphatase, and calcium data for first passage cultures were evaluated with one way analysis of variance (ANOVA) after the data passed normality and equal variance tests. All pairwise multiple comparisons were conducted with the Student-Newman-Keuls method. Comparisons between cells in control and OS medium for these assays were done with the *t*-test.

RESULTS

Morphology

Rat marrow-derived MSCs maintained in either 20% or 5% oxygen in primary culture were essentially identical as viewed by phase contrast microscopy. Small colonies of fibroblast-like cells were seen after several days of primary culture (Fig. 2A). These colonies increased in size and were subcultured after 12–14 days. Colonies appeared to be larger and were significantly more numerous in cultures maintained in low oxygen (Fig. 3). Individual cells, however, appeared to be identical in either oxygen condition in these primary colonies. The pH of culture medium collected when medium was changed (days 3, 7, 11, and 13) was statistically identical for rMSC cultures maintained in the two oxygen environments (data not shown). Individual subcultured cells in control medium were also similar to one another in morphology regardless of the oxygen condition (not shown). First passage cells were more uniformly distributed on the culture substrate and were somewhat more spread than in primary culture.

When cultured in OS medium in first passage, rMSCs assumed a less elongated, polygonal appearance. By approximately day 7, small foci of small round cells, presumed to be osteoblasts, could be seen at multiple sites among confluent fibroblast-like cells throughout the cultures (Fig. 2B). These foci, referred to as nodules, increased in size and number through time in culture, becoming multilayered (Fig. 2C) and then mineralizing as confirmed by brown to black von Kossa staining.

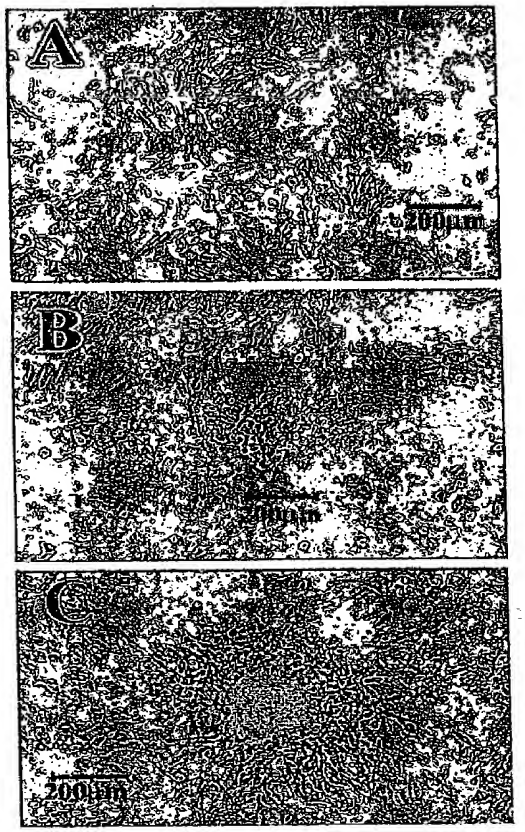

Fig. 2. A: A colony of rMSCs on day 8 of primary culture. B: rMSCs on day 8 of first passage. The presence of osteogenic supplements in the culture medium (OS medium) promotes the formation of bone nodules. A small nodule in the early stage of development can be seen in the center of the field. C: A larger, multilayered, mineralizing nodule on day 13 of first passage in OS medium. All photomicrographs are of cultures in low oxygen; cultures in control oxygen (not shown) are comparable in morphology. Bars in each panel indicate scale.

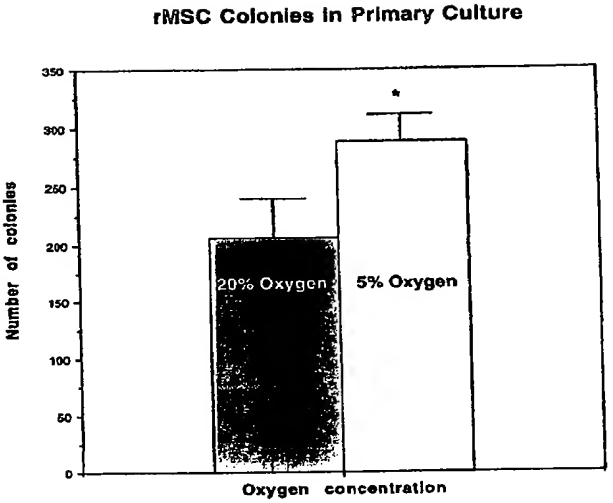

Fig. 3. The number of rMSC colonies on day 7 of primary culture in control oxygen (solid column) or low oxygen (open column) is represented on the Y axis. Values indicate the mean and standard deviation for nine cultures for each condition; all cultures were from the same cell preparation. *Significantly higher ($P < 0.0001$).

Internodular cells maintained their polygonal morphology, became multilayered, and were negative for von Kossa staining; these morophologies are as previously reported by us and others (Herbertson and Aubin, 1995; Cassiede et al., 1996; Hanada et al., 1997). First passage cells in control medium (which contained ascorbic acid-2-phosphate and BGP but not dexamethasone) were more elongated than the non-nodular cells in OS medium. The cells in control medium became multilayered, but did not form nodules and were always von Kossa-negative.

Cell proliferation

For each of five separate rMSC preparations, the number of cells harvested at first passage, as determined by hemacytometer count, was higher for cultures maintained in low oxygen than for those in control oxygen (Table 1). When these numbers were analyzed with Student's paired t-test, the difference was found to be significant ($P < .01$). The effect of oxygen concentration was also evaluated by determining the percent change in cell yield for each preparation (Table 1). Thus, a positive change (increase) in cell number for rMSCs cultured in low oxygen was calculated. When the data from all preparations were considered, the mean increase in cell number for rMSCs cultured in low oxygen was determined to be 41.2% (±18.8%).

DNA content per culture was calculated to evaluate differences in cell number among first passage cultures

TABLE 1. Effect of oxygen concentration on rMSC yield at first passage

Preparation	Control oxygen cell yield ($\times 10^6$)	Low oxygen cell yield ($\times 10^6$)*	% Increase in cell yield for rMSCs in low oxygen**
1	1.24	1.93	55.7
2	2.18	3.17	45.4
3	1.59	2.51	57.9
4	1.46	1.97	34.9
5	2.01	2.25	11.9

Cell yield per 100-mm culture dish at first passage is illustrated for rMSCs cultured in control (20%) and low (5%) oxygen. Cell numbers were determined by hemacytometer count.
*Significantly higher ($P < 0.01$) than cell yield for rMSCs maintained in control oxygen when data are analyzed with the paired t-test.
**Data indicate the difference in yield for rMSCs maintained in the two conditions according to the following formula (after Pennathur-Das and Levitt, 1987)

$$\frac{\text{Cells/plate in 5\% oxygen} - \text{Cells/plate in 20\% oxygen}}{\text{Cells/plate in 20\% oxygen}} \times 100$$

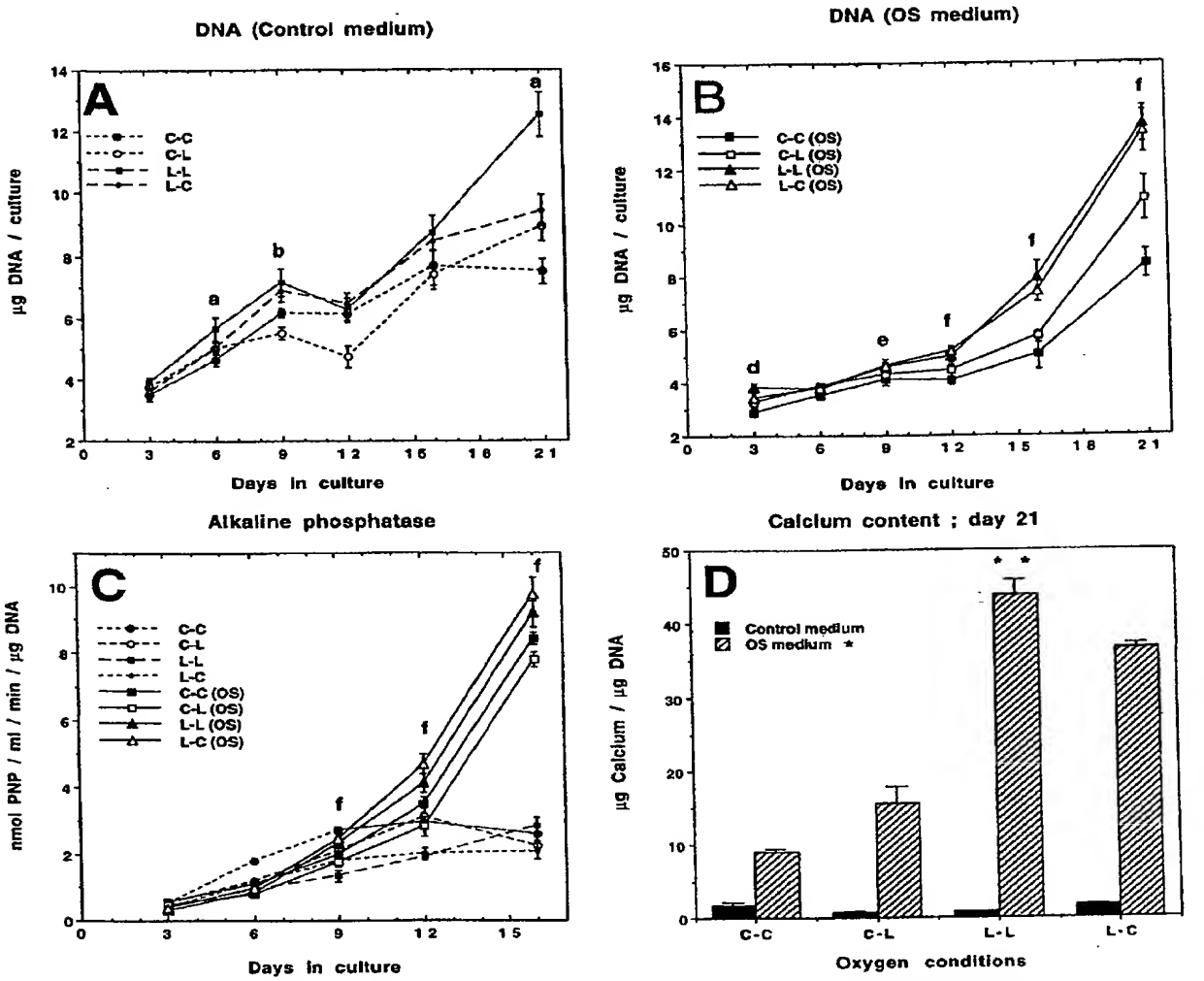

Fig. 4. A and B: Time course for DNA content per culture for first passage rMSCs in control medium (A) and in OS medium (B) in four different oxygen conditions. Control medium is DMEM-LG supplemented with 10% FBS and 80 µM ascorbic acid-2-phosphate plus 10 mM β-glycerophosphate starting on day 10. OS medium contains 10^{-7} M dexamethasone in addition to the other components. Statistically significant data ($P = 0.05$ or less) are indicated by lower case letters as follows: (a) L-L is higher than other groups in control medium; (b) L-L is higher than C-C and C-L in control medium; (d) L-L is higher than other groups in OS medium; (e) L-L and L-C are higher than C-C; (f) L-L and L-C are higher than C-C and C-L. C: Time course for alkaline phosphatase activity per µg DNA for first passage rMSCs in different oxygen conditions in control medium (dashed lines) or OS medium (solid lines). (f): L-L and L-C are significantly higher ($P = 0.05$) than C-C and C-L in OS medium. D: Calcium content per µg DNA in first passage rMSCs in control medium (solid columns) or OS medium (hatched columns) in different oxygen conditions on day 21 of culture. (*) Calcium per µg of DNA for all groups in OS medium is significantly higher ($P = 0.03$ or less) than for the same oxygen conditions in control medium; (**) Significantly higher ($P < 0.0001$) than for other groups in OS medium. For all panels, data points and error bars represent the mean and standard deviation for triplicate cultures. Designation of oxygen conditions is the same as in Figure 1.

in control or OS medium in the four oxygen conditions (Figs. 4A, B). The data depicted in Figure 4 are derived from a single preparation of rMSCs, and are representative of the data from five separate rMSC preparations. As illustrated in Figure 4A, the amount of DNA per culture for cells in control medium increased from day 3 to 21 except for a slight decline at day 12. On most days, DNA values were higher for cells which had been in low oxygen in primary culture (L-L and L-C) than for those in control oxygen in primary culture (C-C and C-L). On

days 6 and 21, DNA values for those in low oxygen in both passages were significantly higher ($P < .05$) than those for any of the other groups.

Rat MSCs in osteoinductive (OS) medium had lower quantities of DNA than their sister cultures in control medium except on day 21 (Fig. 4B). As in the case of rMSCs in control medium, DNA for cells in OS medium increased through time in culture. There was, however, no decline between day 9 and 12, and the increase in DNA after day 12 was greater than for control medium. Cells in L-L and L-C conditions in OS medium were not significantly different from one another except on day 3, but both had higher DNA values than for those in C-C and C-L conditions on all assay days except day 6; in most cases the differences were significant. C-L cultures in OS medium had higher DNA values than those in C-C conditions.

Thus, oxygen tension affected cell proliferation both in primary culture and in first passage. Cell numbers were higher at the end of primary culture for cells in low oxygen than for those in control oxygen. Rat MSCs continued in low oxygen in first passage (L-L) had higher DNA values than those continued in control oxygen (C-C). When data from all preparations are considered (not all data shown), the number of cells was slightly lower, on most days on which they were evaluated, for rMSCs in L-C conditions than for those in L-L conditions. Conversely, cell numbers were somewhat higher for C-L cultures than for those in C-C conditions. That is, switching cultures from low to control oxygen at first passage resulted in lower cell numbers, while switching from control to low oxygen resulted in higher cell numbers.

Alkaline phosphatase activity

While the data depicted in Figure 4C are representative of the effect of oxygen concentration on alkaline phosphatase (AP) activity (normalized per μg of DNA) for rMSCs, there was a greater degree of variability from one preparation to another than in the case of DNA. In most instances, as shown in Figure 4C, AP activity was higher in cells maintained in control medium than in OS medium, through day 6, regardless of the oxygen environment. By day 9, however, the levels of AP activity in rMSCs in OS medium usually exceeded those for cells in control medium. AP activity for rMSCs in control medium rose slightly from day 9 to 12, and then declined by day 16, except for cells in the L-L and L-C groups. On the other hand, AP activity of rMSCs in OS medium increased approximately two-fold from day 9 to 12 and then doubled again from day 12 to 16.

With respect to first passage rMSCs in OS medium, L-L and L-C cultures were approximately equivalent in AP activity on day 9 and thereafter, and C-C and C-L cultures were comparable to one another in the same interval. AP activity was significantly higher ($P = 0.05$) for the former groups. Although L-C cultures had higher AP/DNA values than L-L cultures, the differences, except for day 12, were not significant.

Calcium content

For each of five separate preparations of rMSCs, day 21 calcium content, normalized per μg of DNA, was greater for cultures in OS medium than for those in control medium, regardless of the oxygen concentration in which the cells were maintained. There was a clear correlation between calcium content and oxygen concentration for rMSCs maintained in OS medium. Calcium content per μg of DNA on day 21 was highest in cells in L-L conditions and lowest in cultures in C-C conditions (Fig. 4D). Moreover, the influence of oxygen tension was also evident in the calcium values of cells switched from one oxygen condition to the other at first passage. Cultures transferred from low to control oxygen had lower calcium values than those continued in low oxygen, while those transferred from control to low oxygen had higher calcium content than those maintained continuously in control oxygen.

von Kossa staining

Staining of rMSCs by the von Kossa procedure visually demonstrated that oxygen tension affected the extent of mineralized bone nodule formation in first passage cultures in OS medium in the same manner that it influenced calcium content. The number of mineralized nodules was much greater in cultures in L-L and C-L conditions than for those in C-C conditions (Fig. 5).

In vivo osteogenic assay

Histologic evaluation of porous ceramic cubes harvested from syngeneic host animals 3 and 6 weeks after implantation revealed that greater amounts of bone and cartilage are present in cubes loaded with rMSCs from primary cultures cultivated in low oxygen than in those containing cells cultured in control oxygen in primary culture. The results for all ceramic cubes harvested at 6

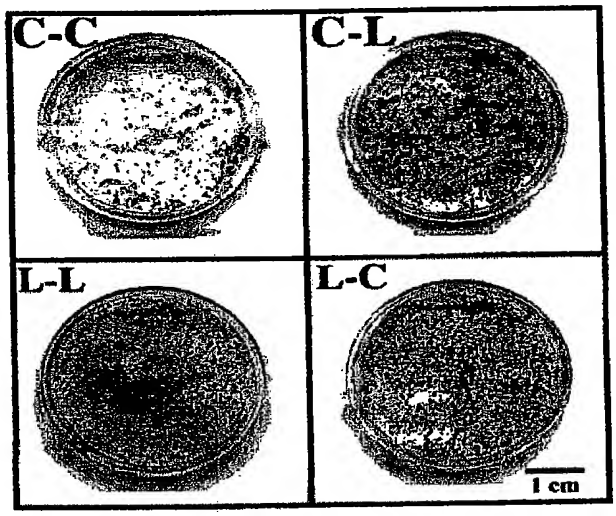

Fig. 5. Cultures of rMSCs (in 35-mm tissue culture dishes) maintained in different oxygen conditions were fixed on day 21 of first passage and stained by the von Kossa method to reveal mineralization. Designation of oxygen conditions (C-C, etc.) is the same as in Figure 1. All cultures were maintained in OS medium. Each focus of stained cells corresponds to an individual bone nodule or group of fused nodules.

TABLE 2. Effect of oxygen concentration in primary culture on bone formation in implanted rMSC-loaded porous ceramic cubes

Cell preparation (1–5); host animal (A or B)	Percentage of bone or cartilage-containing pores in cubes loaded with	
	Cells cultured in control oxygen	Cells cultured in low oxygen*
1A	9.6	35.8
1B	27.0	43.8
2A	51.2	61.4
2B	53.2	69.1
3A	0.36	7.5
3B	0.23	35.8
4A	36.4	70.9
4B	38.3	75.4
5A	64.1	81.1
5B	44.3	69.1

Rat MSCs from five separate preparations (indicated by numbers 1–5) cultured in control or low oxygen were loaded into porous ceramic cubes which were implanted into two syngeneic host animals (designated by the letter A or B) per preparation. Cubes were harvested after six weeks and evaluated for the presence of bone and cartilage. The scores illustrate the number of bone- or cartilage-containing pores as a percentage of the total number of pores and are expressed as the mean for four cubes per oxygen condition per host animal.
*Significantly higher ($P = 0.0003$) than scores for cells in control oxygen when the data are analyzed with the paired t-test.

Fig. 6. Photomicrographs of representative histologic sections of rMSC-loaded porous ceramic cubes in which the development of bone (A) or cartilage (B) has occurred. A: Section of a cube which had been loaded with rMSCs, implanted into a syngeneic host, harvested after 3 weeks, and then fixed, embedded, sectioned, and stained with toluidine blue. In several pores in this section bone (b) has been fabricated by osteoblasts (ob) on the walls of the pores. The black arrows point to three osteoblasts among a large number of osteoblasts actively forming bone. Osteocytes (black arrowheads) are encased within the bone matrix. Vascularized fibrous tissue (f) fills the center of the pore. Ceramic material has been extracted during demineralization of the sample, leaving a whitish gray residue referred to as ceramic ghost (cg). B: Cartilage (c) is present in two pores of a cube harvested at 6 weeks after implantation; cartilage matrix in the pore on the left has stained more intensely with toluidine blue. Cartilage in a third pore (e) has been resorbed by invading host vasculature and is being replaced by bone (white arrowheads). Other labels are the same as for panel A. Cartilage-containing pores comprise only 5–10% of all pores considered to be bone- or cartilage-positive. The two panels demonstrate typical bone and cartilage formation, but, while harvested at different times after implantation, are not intended to represent a typical temporal sequence of histologic development. Moreover, although both samples depict sections of cubes loaded with rMSCs cultured in low oxygen, cubes loaded with rMSCs cultivated in control oxygen (not shown) are qualitatively similar in morphology. Scale is indicated by bars in each panel.

weeks are presented in Table 2. For each of the ten individual host animals (two rats for each of five separate cell preparations), the mean score (expressed as the percentage of pores which contained bone or cartilage) for cubes loaded with cells cultured in 5% oxygen was higher than that for cubes containing cells maintained in 20% oxygen. In most instances, four cubes were implanted per animal for each of the two oxygen conditions.

There was, however, no qualitative histologic difference between the two sets of cubes. In either case, osseous material was initially deposited along the inner walls of bone-containing pores by cubodial cells presumed to be osteoblasts (Fig. 6A). Some osteoblasts became enclosed within the bony matrix (becoming osteocytes in the process) as deposition of bone progressed uniformly toward the center of the pore. Varying amounts of fibrous tissue and vascular elements were also located within bone-containing pores. In some cases, entire pores were filled with cartilage, identified by large round to cubodial cells surrounded by matrix which stained metachromatically with toluidine blue (Fig. 6B). Cartilage-containing pores represented a small portion of pores identified as bone- or cartilage-positive. Pores which contained neither bone nor cartilage were filled with vascularized fibrous tissue.

DISCUSSION

In this study, rat marrow-derived MSCs were cultivated in standard tissue culture conditions and in an atmosphere of reduced oxygen in order to determine whether the two conditions differ in their effect on cell attachment and proliferation and on osteochondrogenesis. The results suggest, first, that attachment and/or survival of rMSCs in primary culture is enhanced in a reduced oxygen environment, as indicated by cell colonies which were significantly higher in number than for those maintained in control oxygen. Secondly, a greater number of cells per dish were harvested at first passage from cultures maintained in 5% oxygen, suggesting that the rate of cell proliferation in primary culture was higher in low oxygen. While it is possible that the increase in cell number resulted solely from the greater number of colonies, examination of the cultures by phase contrast microscopy suggests that colonies in cultures maintained in low oxygen are, on average, larger in size and not just greater in number than those in control oxygen, although the difference in colony size was not quantified.

Moreover, elevated rates of proliferation, independent of any influence of colony number, continued in first passage for rMSCs which had been in 5% oxygen in primary culture and first passage, as indicated by higher DNA values for these cells in both control and OS medium. Changing oxygen concentration at first passage almost always resulted in less of an increase in DNA, over time, for cells switched from low to control oxygen, and in a greater increase in DNA for cells transferred from control to low oxygen (compared, in each case, with those for which oxygen tension was not changed).

Evidence for the notion that in vitro osteogenesis is enhanced in an atmosphere of reduced oxygen tension is found in data regarding calcium content and von Kossa staining of bone nodules. For each of the five preparations of rMSCs tested, calcium content was elevated in all cultures which were maintained in low oxygen during at least one passage in vitro, relative to those in control oxygen throughout their time in culture. Moreover, von Kossa staining of selected rMSC cultures from each cell preparation revealed that more nodules are present in those maintained continuously in low oxygen than in control oxygen. Bellows and Aubin (1989) have demonstrated that individual nodules are derived from single osteoprogenitors in cultures of fetal rat calvarial cells. Assuming that this observation also applies to MSC cultures, we conclude that an atmosphere of low oxygen favors proliferation of mesenchymal stem or osteoprogenitor cells and maintenance of the stem or osteoprogenitor phenotype.

It is possible that commitment of rMSCs along osteogenic lines is more likely to be initiated in an atmosphere of reduced oxygen tension in primary culture, and that increased expression of osteogenic markers may then occur, but only when the cells are placed in an osteoinductive environment. Two observations support this contention. First, rMSCs cultured in reduced oxygen tension in primary culture produced more bone and cartilage when loaded into fibronectin-coated ceramic cubes and implanted subcutaneously into syngeneic hosts than do those cultured in control oxygen. Secondly, when primary cultures of rMSCs maintained in low oxygen in a medium (control medium) which does not favor differentiation were switched to control oxygen and osteogenic medium at first passage, calcium values were higher than for rMSCs in the same medium conditions but in control oxygen in both passages. It is important to note that calcium values were low in all oxygen conditions in the absence of osteogenic supplements in the culture medium. Thus, low oxygen alone may predispose rMSCs to commit to the osteogenic lineage, but, in the absence of the appropriate stimuli, elevated expression of osteogenic markers is not observed.

On the other hand, elevated calcium and alkaline phosphatase levels and increased von Kossa-positive staining were also observed in rMSCs maintained in control oxygen in primary culture but switched to low oxygen and OS medium at first passage. Thus, even in the absence of putative pre-induction by reduced oxygen tension in primary culture, the osteogenic expression of rMSCs is enhanced in the presence of low oxygen in first passage. It may be that low oxygen favors both commitment to osteogenic lines and differentiation along those lines, but also favors osteogenic differentiation even in the absence of prior enhanced commitment.

Other investigators have examined the effect of reduced oxygen tension on osteoblasts (Tuncay et al., 1994; Reilly et al., 1998), pericytes (Brighton et al., 1992; Reilly et al., 1998), and periosteal cells (Deren et al., 1990). Like rMSCs, periosteal cells and pericytes are thought to have the potential to differentiate into osteoblasts given the proper environmental cues. It is interesting to note, therefore, that the rates of proliferation and the alkaline phosphatase activity of these cells are elevated in a low oxygen environment. On the other hand, alkaline phosphatase activity of rat calvarial osteoblasts was higher in 90% oxygen than in either 10 or 21% oxygen (Tuncay et al., 1994). Additionally, levels of osteocalcin, thought to be a late marker of osteoblastic expression, were higher in cultures of pericytes at 7 weeks of culture in 21 and 60% oxygen than in lower oxygen tensions (Brighton et al., 1992; Reilly et al., 1998). While osteocalcin levels were not examined in the present study, calcium content, bone nodule formation, and in vivo osteogenesis, other late markers of osteogenic expression, were found to be elevated in reduced oxygen in rMSCs. These parameters, however, were not examined as late as seven weeks of culture, and it is difficult to make comparisons among these various studies because of differences in cell type, species, medium, serum, and the timing of the assays.

The low oxygen mixture employed in this study differs from the atmosphere customarily used in cell culture not only in oxygen concentration, but also in elevated nitrogen tension and in the absence of argon, neon, and other gases present in the atmosphere in trace quantities. While it is possible, though we believe unlikely, that these non-oxygen related differences affected the experimental results, to our knowledge, no data are available regarding the impact of these atmospheric constituents on cell culture. While human MSCs were not examined under the conditions described in this paper, it is conceivable that they may be affected by lower oxygen tension in the same manner as for rMSCs. If that were the case, cultivation of human MSCs in low oxygen could play an important role in possible clinical application of these cells.

Finally, a review of the literature reveals a broad range of cell types (beyond the brief list presented in the Introduction) for which cultivation in physiologic oxygen has been found to be superior to that in conditions normally found in vitro. Perhaps the oxygen concentration to be employed in the culture of a given cell type should be accorded as much consideration as is given to the choice of culture medium or serum. It seems fair to say that, while the use of 95% air (20% oxygen) is sufficient to observe osteogenic events in vitro, this oxygen level is neither optimal nor physiologic.

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